*J. Pineal Res. 2003; 35:276–282*


**Journal of Pineal Research**

# Protective effect of indoleamines on in vitro ascorbate-$Fe^{2+}$dependent lipid peroxidation of rod outer segment membranes of bovine retina

Abstract: Rod outer segment membranes (ROS) are highly vulnerable to autooxidation because of their high content of long chain polyunsaturated fatty acids (PUFAs). Melatonin and N-acetylserotonin are indoleamines synthesized in the pineal gland, retina and other tissues. These compounds are free radical scavengers and indirect antioxidants because of their stimulatory effect on antioxidative enzymes. We compared the in vitro protective effect of melatonin and N-acetylserotonin on the ascorbate-$Fe^{2+}$ induced lipid peroxidation of PUFAs located in ROS membranes. This process was measured by chemiluminescence and fatty acid composition of total lipids of ROS. We assayed increasing concentrations of melatonin (0–10 mM) and N-acetylserotonin (0–2 mM). In both cases the total cpm originated from light emission (chemiluminescence) was found to be lower in those membranes incubated in the presence of either melatonin or N-acetylserotonin; this decreased proportional to the concentration of the indole. Thus, 10 mM melatonin and 2 mM N-acetylserotonin produced a reduction of 51 ± 6 and 100% in the total chemiluminescene (lipid peroxidation), respectively. We also noticed a PUFAs protection: the docosahexaenoic acid content decreased considerably when the membranes were submitted to oxidative damage. This reduction was from 37.6 ± 2.1% in the native membranes to 6.2 ± 0.8% in those which were peroxidized. These changes were less pronounced in treated ROS membranes; as an example in the presence of 10 mM melatonin or 2 mM N-acetylserotonin we observed a content preservation of 22:6 n-3 (23.6 ± 1.2 and 39.1 ± 1.2% respectively). The concentration of each compound required to inhibit 50% of the lipid peroxidation ($IC_{50}$) was 9.82 mM for melatonin and 0.43 mM for N-acetylserotonin, respectively. N-acetylserotonin shows a protective effect about 20 times higher than that of melatonin.

**Margarita H. Guajardo, Ana M. Terrasa and Angel Catalá**

Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina

Key words: lipid peroxidation, melatonin, N-acetylserotonin, retina, rod outer segments

Address reprint requests to Angel Catalá, Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC296, B1900 AVW, La Plata, Argentina.
E-mail: acatala@fcv.unlp.edu.ar

Received May 19, 2003;
accepted August 4, 2003.

## Introduction

Rod outer segment membranes (ROS) of bovine retina are susceptible to lipid peroxidation because of their high content of polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (22:6 n-3) [1]. It has been suggested that lipid peroxidation participates in the oxidative damage leading to retinal cell degeneration. Oxidative stress has been discussed as a possible risk factor for eye diseases such as glaucoma, cataract, and retinal damage. Oxidative stress is defined as an imbalance of prooxidants (reactive oxygen species including free radicals) and antioxidants [2].

Lipid peroxidation proceeds by a chain reaction that includes initiation, propagation and termination. Initiation occurs when an oxidant gives rise to an initiating lipid peroxyl radical ($LOO^{\cdot}$) by reaction with either a lipid (LH) or pre-existing lipid hydroperoxide (LOOH). Propagation is cycled through rounds of $LOO^{\cdot}$ abstraction of the bis-methylene hydrogen atoms of a polyunsaturated fatty acyl chain to generate additional $LOO^{\cdot}$ (after $O_2$ addition) which results in the net conversion of lipids to LOOHs. Lipid peroxidation termination involves the reaction of two $LOO^{\cdot}$ to form non-radical products or the reaction of one $LOO^{\cdot}$ with another terminating radical to generate non-propagating radical species [3]; the first reaction is particularly interesting as it is accompanied by emission of chemiluminescence [4]. The requirement of iron in lipid peroxidation has been demonstrated by Minotti et al. [5].

Antioxidants are essential to photoreceptor survival because these cells are characterized by high-energy demand, which is associated with the mitochondrial production of partially reduced oxygen species [6]. Melatonin and tryptophan derivatives such as N-acetylserotonin possess free radical scavenging activity. Experimental studies have shown that melatonin directly scavenges the hydroxyl radical, peroxynitrite anion, and secondarily

scavenges superoxide anion and quenches singlet oxygen [7]. Furthermore, this tryptophan derivative stimulates a number of antioxidative enzymes and stabilizes cell membranes [8]. Melatonin is produced in a limited number of organs in mammals including the pineal gland, retina and in the gastrointestinal tract. There are no morphophysiological barriers to melatonin because its solubility in both lipid and water allows it to pass all barriers and to quickly enter all cells and cellular organelles where it provides protection of lipids, proteins and DNA [9].

In the present study we investigated the ability of melatonin and N-acetylserotonin to protect against ascorbate-$Fe^{2+}$-induced oxidative damage in bovine ROS. To test the antioxidant effect of these indoleamines, co-incubation with different concentrations of melatonin or N-acetylserotonin varying from 0 to 10 mM and 0 to 2 mM, respectively, were used.

## Materials and methods

Bovine eyes were obtained from Gorina slaughterhouse (La Plata, Argentina). Butylated hydroxytoluene (BHT), phenyl methylsulfonyl fluoride (PMFS), bovine serum albumin (BSA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fatty acids and standards of fatty acid methyl esters were from Nu Chek Prep. (Elysian, MN, USA). L (+) ascorbic acid was from Merck (Buenos Aires, Argentina). Boron trifluoride-methanol complex (14%), melatonin (N-Acetyl-5-methoxytryptamine) and N-acetylserotonin (N-Acetyl-5-hydroxytryptamine) were from Sigma (St Louis, MO, USA). All other reagents and chemicals were of analytical grade.

### Isolation of bovine ROSs

Bovine ROSs were obtained from retinal homogenates by a method described by De Gripp et al. [10], using a continuous sucrose density gradient. Eyes were enucleated at slaughter, transported on ice to the laboratory where retinas were dissected within 1–2 hr. The isolation procedure was conducted in red light and with all tubes and solutions were kept on ice, corneas were excised; the lens and vitreous were subsequently removed. Eyecups were inverted and retinas were carefully peeled from the eyes. Fifteen retinas were briefly homogenized in physiological solution (1 mL/retina) for 20 s at 4°C in a Potter–Elvehjem homogenizer. After filtration through a very thin stainless-steel mesh, the filtrate was mixed with 1.28 M sucrose solution to a final concentration of 0.42 M. From this suspension and a 1.28 M sucrose solution, a continuous gradient with a density range of 1.05–1.16 g/mL was prepared. A red band containing ROSs in the middle of the tubes was obtained after centrifugation at 13,200 × g for 90 min. ROS were recovered by aspirating the layer from the top and then purified with five volumes of 0.15 M NaCl. Then, they were centrifuged at 1000 × g for 20 min and the pellet resuspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM PMFS. The preparation was stored at −84°C to minimize possible autooxidation of lipid components.

### Protein determination

Protein concentration of the ROS membranes was determined by the method of Lowry et al. [11] using BSA as standard.

### Lipid peroxidation and chemiluminescence assay

The ROS membranes (0.25 mg of protein) were incubated at 37°C in 0.05 M phosphate buffer pH 7.4, final volume 2 mL. Lipid peroxidation was started by addition of ascorbate (final concentration 0.4 mM). Phosphate buffer contained sufficient iron to provide the necessary ferrous or ferric iron for lipid peroxidation (final concentration in the incubation mixture was 2.15 μM). Stock solutions of N-acetylserotonin (0.2 M) and melatonin (0.5 M) were prepared in ethanol. Simultaneously treated membranes containing increasing concentrations of melatonin (5, 6.25, 7.5, 8.75 and 10 mM) or N- acetylserotonin (0.5, 1, 1.5 and 2.0 mM) were assayed. In all the cases, controls without ascorbate were carried out. Lipid peroxidation was measured by monitoring light emission [12] with a liquid scintillation analyzer Packard 1900 TR (Meriden, CT, USA). Chemiluminescence was determined over a 180-min period and recorded as cpm every 10 min to establish the time course of lipid peroxidation. The sum of the total cpm was used to compare the inhibitory effect produced by indoleamines.

### Lipid extraction and fatty acid analysis

Lipids from native and peroxidized ROS membranes were extracted with chloroform:methanol (2:1 v/v) containing 0.01% BHT as antioxidant, according to the method of Folch et al. [13]. Fatty acids were transmethylated with $BF_3$, in methanol at 60°C during 180 min and the fatty acid methyl esters were extracted with hexane:water (2:1 v/v). A 2-μL aliquot was injected through the column injector of GC-14 A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a packed column (1.80 m × 4 mm id) GP 10% DEGS-PS on 80/100 Supelcoport. Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250°C. The column temperature was held at 200°C during 60 min. Comparing retention times with standards, the fatty acid methyl ester peaks were identified. All compositions were expressed as % by area of total fatty acids.

### Statistical analysis

All data are represented as mean ± S.E.M. of three independent determinations. Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey test. The statistical criterion for significance was selected at different $P$ values, which were indicated in each case.

## Results

When ROSs (0.25 mg protein) were incubated at 37°C in the presence of ascorbate-$Fe^{2+}$ a time-dependent increase

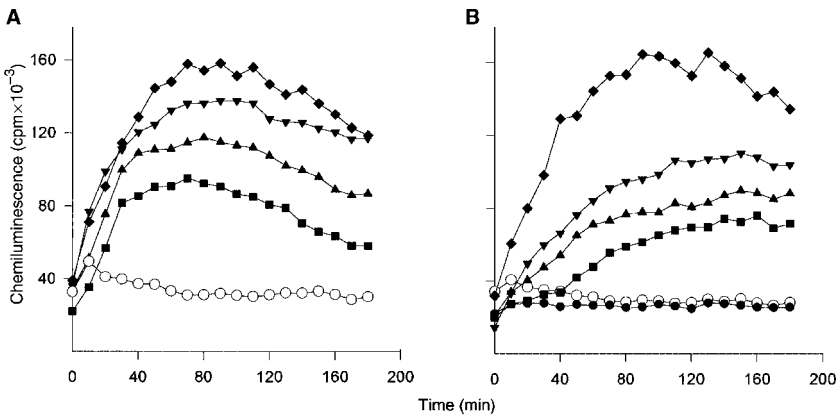


*Fig. 1.* Chemiluminescence as a function of time during $Fe^{2+}$-ascorbate lipid peroxidation of rod outer segment membranes (0.25 mg of protein). (A) Effect of melatonin, (–○–) control without ascorbate; –■– 10 mM; –▲– 7.5 mM; –▼– 5 mM and –◆– 0 mM melatonin. (B) Effect of N-acetylserotonin, (–○–) control without ascorbate; –●– 2 mM; –■– 1.5 mM; –▲– 1 mM; –▼– 0.5 mM and –◆– 0 mM N-acetylserotonin.

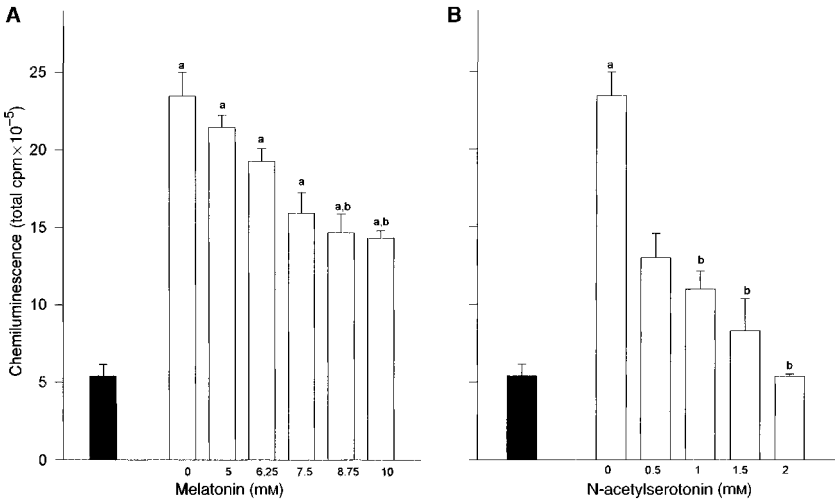


*Fig. 2.* Total chemiluminescence produced by rod outer segment membranes (0.25 mg of protein) recorded as cpm every 10 min during 180 min of incubation as a function of indoleamine concentration. Effect of melatonin (A) and N-acetylserotonin (B). □ With 0.4 mM ascorbate, ■ without ascorbate. Data are mean ± S.E.M. of three independent experiments. $^{a}P < 0.001$ indicates a significant difference between control without ascorbate and all the peroxidized groups. Statistically significant differences between peroxidized versus peroxidized + indoleamine groups are indicated by $^{b}P < 0.001$.

in chemiluminescence was measured. Fig. 1 shows lipid peroxidation experiments in which the two different indoleamines were assayed. Chemiluminescence as a function of time was measured for 180 min in ROS membranes with and without 0.4 mM ascorbate. The same procedure was applied to ROS membranes previously incubated with different concentrations of melatonin ranging from 5 to 10 mM and N-acetylserotonin from 0.5 to 2 mM. The addition of ascorbate produced a considerable increase of chemiluminescence; peaks of maximum light emission (about 160,000 cpm) at 90 min were observed in both experiments (Fig. 1A, B). The addition of different concentrations of melatonin or N-acetylserotonin to the incubation mixture diminished the lipid peroxidation elicited by ascorbate-$Fe^{2+}$ in a concentration-dependent fashion.

Figure 2 shows the total cpm originating from chemiluminescence, measured every 10 min, during 180 min of incubation of ROS membranes with different amounts of melatonin (Fig. 2A) and N-acetylserotonin (Fig. 2B). The addition of indoleamines to the ROS membranes produced inhibition of the chemiluminescence, depending on the concentration used. The ability of indoelamines to reduce chemiluminescence in ROS membranes in the presence of a metal catalyzed oxidation system, ascorbate-$Fe^{2+}$, was N-acetylserotonin > melatonin. The inhibitory effect was indoleamine concentration-dependent. In the absence of ascorbate the total chemiluminescence was $5.39 \pm 0.76 \times 10^5$ cpm, whereas in the presence of ascorbate, this value was incremented to $23.44 \pm 1.54 \times 10^5$ cpm. When increasing amounts of melatonin were added the light emission was decreased proportionally to the concentration, thus at 10 mM melatonin the total chemiluminescence was $14.32 \pm 0.44 \times 10^5$ cpm. The total chemiluminescence was greatly reduced to $5.41 \pm 0.14 \times 10^5$ cpm, with 2 mM N-acetylserotonin. At this concentration the total chemiluminescence was similar to the control (without ascorbate), with antioxidant protection of 100%; for 10 mM melatonin, the percent of inhibition was 50.89 ± 5.96. Statistically significant differences between native versus peroxidized with or without melatonin were observed. No important differences in the chemiluminescence values between control without ascorbate and N-acetylserotonin treated groups were observed. When we compared samples peroxidized in the absence of indoleamines with treated groups, statistically significant differences at 8.75 and 10 mM melatonin and from 1 to 2 mM N-acetylserotonin were observed.

Figure 3 shows the inhibitory effect of melatonin or N-acetylserotonin on $Fe^{2+}$-ascorbate induced lipid peroxidation in ROS membranes. The $IC_{50}$ was determined by calculating the concentration of each indoleamine required

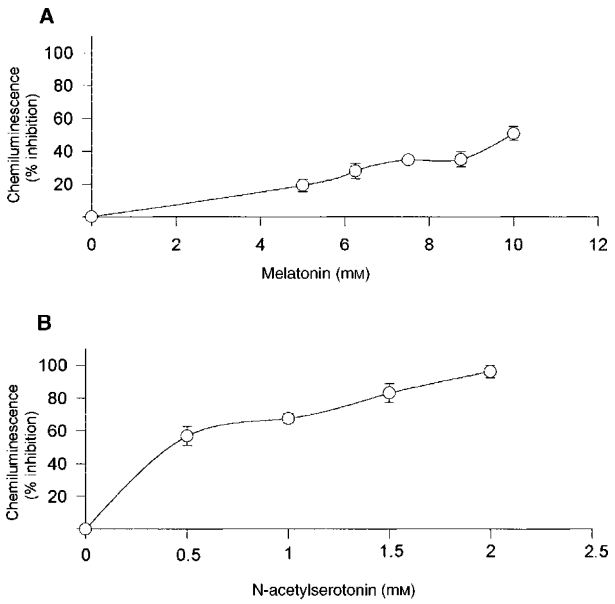


*Fig. 3.* Inhibition percent of total chemiluminescence by melatonin (A) and N-acetylserotonin (B). The zero percent of inhibition was calculated by subtracting the total cpm originated by control without ascorbate from peroxidized rod outer segment membranes (with ascorbate). The percent of inhibition for each indoleamine concentration was calculated by comparing the total cpm with the zero percent.

to inhibit 50% of the light emission (lipid peroxidation). The efficacy of N-acetylserotonin to inhibit the lipid peroxidation was greater than melatonin; thus, the $IC_{50}$ for N-acetylserotonin was 0.43 mM whereas the $IC_{50}$ for melatonin was 9.82 mM.

Changes in the fatty acid profiles were used as an index of the oxidative damage to lipids. The long-chain fatty acid mainly affected during the lipid peroxidation process was the PUFA 22:6 n-3 which decreased considerably. As a consequence, they brought about a relative increase in the percentage of saturated and monoenoic fatty acids.

Table 1 summarizes the fatty acid composition of total lipids from ROS membranes:native (control) and peroxidized with 0, 5, 6.25, 7.5, 8.75 and 10 mM melatonin. Statistically significant differences in the content of 20:4 n-6 between native and peroxidized membranes were not observed. However, statistically significant differences ($P < 0.001$) in the content of 22:6 n-3 between native versus peroxidized with 0, 5 and 6.25 mM melatonin was found. These changes were less pronounced ($P < 0.01$) between native versus peroxidized with 7.5, 8.75 and 10 mM melatonin groups. As an example, 22:6 n-3 decreased from 37.55 ± 2.11% in the native group to 6.23 ± 0.79% after the lipid peroxidation process. Thus, this fatty acid was reduced 83.55 ± 2.14% when ROS membranes were peroxidized in the absence of indoleamines as antioxidants. On the contrary, statistically significant differences ($P < 0.001$) in the content of docosahexaenoic acid were observed when peroxidized and peroxidized + melatonin groups were compared but only from 7.5 to 10 mM melatonin.

The unsaturation index (UI), a parameter dependent of PUFA concentration, was profoundly affected when native and peroxidized groups were compared, statistically significant differences ($P < 0.001$) between native and peroxidized with 0, 5 and 6.25 mM melatonin groups were observed.

Table 2 shows the fatty acid composition of total lipids from ROS membranes (native and peroxidized with 0, 0.5, 1, 1.5 and 2 mM N-acetylserotonin). The columns of native (control) and peroxidized without indoleamine groups are the same in the two tables. Statistically significant differences in the content of 22:6 n-3 between native and peroxidized + N-acetylserotonin groups were not

Table 1. Fatty acid composition of total lipids of native and peroxidized rod outer segment membranes with different melatonin concentration

| Fatty acid | Native (control) | Preoxidized + melatonin (mM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 5 | 6.25 | 7.5 | 8.75 | 10 |
| 16:0 | 14.22 ± 0.97 | 28.61 ± 1.19[a] | 22.98 ± 1.29[b] | 19.26 ± 1.85[d] | 21.37 ± 0.53 | 19.55 ± 1.40[d] | 16.44 ± 2.15[c] |
| 16:1 | 0.16 ± 0.08 | 0.93 ± 0.70 | 0.22 ± 0.11 | 0.35 ± 0.07 | 0.73 ± 0.60 | – | 0.09 ± 0.09 |
| 18:0 | 17.36 ± 0.45 | 23.40 ± 0.66 | 22.22 ± 0.59 | 18.77 ± 1.64 | 20.98 ± 0.52 | 19.50 ± 0.93 | 16.97 ± 1.45 |
| 18:1 n-9 | 8.54 ± 1.35 | 15.11 ± 1.03 | 11.91 ± 2.46 | 11.54 ± 2. 17 | 10.34 ± 0.22 | 10.45 ± 0.50 | 13.85 ± 4.39 |
| 18:2 n-6 | 11.23 ± 1.67 | 19.48 ± 1.60 | 18.38 ± 4.15 | 13.22 ± 5. 51 | 13.65 ± 1.72 | 20.87 ± 1.24 | 22.71 ± 1.29 |
| 20:4 n-6 | 3.93 ± 0.26 | 1.33 ± 0.33 | 2.90 ± 0.33 | 4.65 ± 1.18 | 3.64 ± 0.37 | 2.43 ± 1.25 | 2.89 ± 1.03 |
| 22:6 n-3 | 37.55 ± 2.11 | 6.23 ± 0.79[a] | 17.39 ± 1.72[a] | 20.44 ± 4.72[ad] | 23.52 ± 0.63[bc] | 23.28 ± 1.11[bc] | 23.55 ± 1.20[bc] |
| Saturated | 31.56 ± 0.73 | 52.00 ± 0.54[a] | 45.21 ± 1.81[b] | 38.03 ± 3.47[d] | 42.35 ± 1.02 | 39.05 ± 2.17 | 33.42 ± 3.59[c] |
| Monounsaturated | 8.70 ± 1.40 | 16.03 ± 0.34 | 12.13 ± 2.50 | 11.89 ± 2. 23 | 11.07 ± 0.49 | 10.45 ± 0.50 | 13.93 ± 4.35 |
| Polyunsaturated | 52.70 ± 3.32 | 27.04 ± 2.08[a] | 38.68 ± 5.89 | 38.32 ± 4.44 | 40.81 ± 1.98 | 46.59 ± 1.06[d] | 49.15 ± 1.07[c] |
| Total unsaturated | 61.31 ± 2.02 | 43.07 ± 2.12[b] | 50.81 ± 3.42 | 50.21 ± 2.25 | 51.88 ± 1.49 | 57.04 ± 0.72 | 63.07 ± 3.73[c] |
| Saturated/unsaturated | 0.51 ± 0.01 | 1.21 ± 0.05[a] | 0.90 ± 0.10 | 0.77 ± 0.11[d] | 0.82 ± 0.04[d] | 0.69 ± 0.05[c] | 0.54 ± 0.08[c] |
| UI | 272.15 ± 13.02 | 97.69 ± 6.60[a] | 158.54 ± 22.83[a] | 179.59 ± 17.79[ad] | 194.04 ± 5.61[bc] | 201.63 ± 5.11[c] | 212.21 ± 5.98[c] |

Data shown are given in percentages of total fatty acids content and are mean ± S.E.M. of three separate experiments.
The unsaturation index (UI) was calculated as the sum of the percentage of each fatty acid × the number of olefinic bonds.
Statistically significant differences between native versus peroxidized with and without melatonin groups are indicated by [a]$P < 0.001$ and [b]$P < 0.01$.
Statistically significant differences between peroxidized versus peroxidized + melatonin groups are indicated by [c]$P < 0.001$ and [d]$P < 0.01$ using ANOVA.

Table 2. Fatty acid composition of total lipids of native and peroxidized rod outer segment membranes with different N-acetylserotonin concentration

| Fatty acid | Native (control) | Peroxidized + N-acetylserotonin (mM) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 1 | 1.5 | 2.0 |
| 16:0 | 14.22 ± 0.97 | 28.61 ± 1.19[a] | 20.21 ± 1.57[d] | 19.76 ± 1.12[d] | 14.48 ± 1.34[c] | 15.53 ± 0.23[c] |
| 16:1 | 0.16 ± 0.08 | 0.93 ± 0.70 | 0.53 ± 0.37 | 0.26 ± 0.14 | 0.25 ± 0.12 | – |
| 18:0 | 17.36 ± 0.45 | 23.40 ± 0.66 | 18.01 ± 0.53 | 17.93 ± 1.04 | 16.25 ± 2.11[d] | 17.09 ± 0.43 |
| 18:1 n-9 | 8.54 ± 1.35 | 15.11 ± 1.03 | 7.84 ± 2.06 | 11.48 ± 1.68 | 11.30 ± 2.02 | 6.75 ± 0.53 |
| 18:2 n-6 | 11.23 ± 1.67 | 19.48 ± 1.60 | 22.41 ± 0.85 | 18.50 ± 3.60 | 12.18 ± 5.17 | 12.07 ± 0.65 |
| 20:4 n-6 | 3.93 ± 0.26 | 1.33 ± 0.33 | 2.45 ± 0.09 | 2.67 ± 0.21 | 2.82 ± 0.37 | 3.07 ± 0.33 |
| 22:6 n-3 | 37.55 ± 2.11 | 6.23 ± 0.79[a] | 22.78 ± 2.38[bc] | 25.67 ± 0.93[c] | 35.46 ± 3.52[c] | 39.06 ± 1.23[c] |
| Saturated | 31.56 ± 0.73 | 52.00 ± 0.54[a] | 38.22 ± 1.29[d] | 37.69 ± 1.97[d] | 30.73 ± 3.26[c] | 32.62 ± 0.53[c] |
| Monounsaturated | 8.70 ± 1.40 | 16.03 ± 0.34 | 8.36 ± 1.98 | 11.74 ± 1.76 | 11.55 ± 1.90 | 6.75 ± 0.53 |
| Polyunsaturated | 52.70 ± 3.32 | 27.04 ± 2.08[a] | 47.64 ± 1.63[d] | 46.84 ± 3.58[d] | 50.46 ± 1.41[c] | 54.21 ± 1.64[c] |
| Total unsaturated | 61.31 ± 2.02 | 43.07 ± 2.12[b] | 56.01 ± 2.01 | 58.58 ± 1.88[d] | 62.02 ± 3.20[c] | 60.96 ± 1.99[d] |
| Saturated/unsaturated | 0.51 ± 0.01 | 1.21 ± 0.05[a] | 0.68 ± 0.03[c] | 0.64 ± 0.05[c] | 0.50 ± 0.08[c] | 0.54 ± 0.02[c] |
| UI | 272.15 ± 13.02 | 97.69 ± 6.60[a] | 199.69 ± 12.41[bc] | 213.44 ± 7.16[c] | 259.97 ± 10.36[c] | 277.50 ± 8.76[c] |

Data shown are given in percentages of total fatty acids content and are mean ± S.E.M. of three separate experiments.
The unsaturation index (UI) was calculated as the sum of the percentage of each fatty acid × the number of olefinic bonds.
Statistically significant differences between native versus peroxidized with and without N-acetylserotonin groups are indicated by $^{a}P < 0.001$ and $^{b}P < 0.01$.
Statistically significant differences between peroxidized versus peroxidized + N-acetylserotonin groups are indicated by $^{c}P < 0.001$ and $^{d}P < 0.01$ using ANOVA.

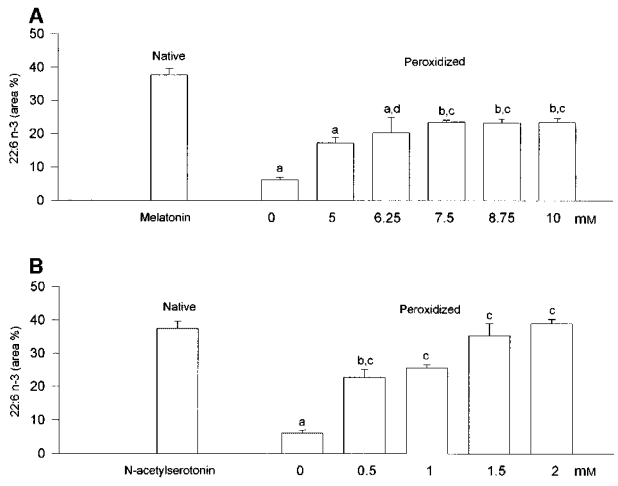


*Fig. 4.* Changes in the docosahexaenoic acid content during the lipid peroxidation. Effect of melatonin (A) and N-acetylserotonin (B). Statistically significant differences between native versus peroxidized with and without indoleamine groups are indicated by $^{a}P < 0.001$ and $^{b}P < 0.01$. Statistically significant differences between peroxidized versus peroxidized + indoleamine groups are indicated by $^{c}P < 0.001$ and $^{d}P < 0.01$ using ANOVA.

observed. However, statistically significant differences ($P < 0.001$) between peroxidized versus peroxidized + N-acetylserotonin groups were observed in all the concentrations assayed.

The content of 22:6 n-3 expressed as area % in native and peroxidized ROS membranes with different amounts of melatonin and N-acetylserotonin is shown in Fig. 4A, B, respectively. The preservation of docosahexaenoic acid by indoleamines during the lipid peroxidation of ROS membranes was observed. The data clearly indicate that the incubation of these membranes in the presence of ascorbate-$Fe^{2+}$ resulted in the peroxidation of membrane phospholipids as evidenced by the loss of 22:6 n-3. At all concentrations of melatonin or N-acetylserotonin tested, docosahexaenoic acid was protected. The protective effect was concentration-dependent. A 50% protection of 22:6 n-3 was reached at 7.5 mM melatonin and 0.5 mM N-acetylserotonin. The inhibition observed in light emission was coincident with the protection of the 22:6 n-3. The 22:6 n-3 content in samples treated with 2 mM N-acetylserotonin was equivalent to that in native membranes which indicated a total protection of this fatty acid.

## Discussion

Peroxidation of PUFAs in lipid bilayer membranes causes loss of fluidity, a fall in membrane potential, increased permeability to protons and calcium ions, and eventually, breakdown of cell membranes because of cellular deformities. The structural and functional integrity of the cell membranes is necessary for signal transduction, molecular recognition and transport, cellular metabolism, etc. The damage inflicted upon biological systems by reactive oxygen species have been implicated in numerous disease processes including inflammation, degenerative diseases, tumor formation and involved in physiological phenomena such as aging [14]. Initiation is the most important phase of lipid peroxidation especially in a cellular context; preventive therapy of lipid peroxidation-associated disease would target the initiation process [3].

Indeed, many ocular disorders including glaucoma, cataracts, diabetic retinopathy and retinal degeneration have been attributed to lipid peroxidation processes [15]. Because of intense exposure to light and oxygen and their high PUFA content which is prone to lipid peroxidation, the retina is highly susceptible to oxidative stress [16, 17].

The retina contains a range of antioxidants that can inactivate free radicals; these antioxidant defenses include enzymes (glutathione peroxidase, superoxide dismutase, catalase) and both lipid and water soluble compounds. The major lipid soluble antioxidants are vitamin E, retinoids and carotenoids. Some aqueous antioxidants include ascorbate, glutathione, taurine, etc [18].

The pineal product melatonin is a powerful antioxidant in many different tissues. It was reported that melatonin prevented lipid peroxidation. Although the mechanism of action of melatonin is not fully understood, some investigators believe that antioxidant properties of melatonin are mediated by its ability to directly scavenge reactive oxygen species [19]. Melatonin was proposed to directly trap superoxide anion and hydroxyl radical in both in vitro and in vivo system [20]. The action of melatonin may involve other mechanisms as well as stabilizing cell membranes [21] and stimulating antioxidative enzymes [22]. Melatonin possesses both hydrophilic and lipophilic characteristics and easily penetrates all biological membranes including the blood–brain barrier [23]. Because of their increased polarity, melatonin may migrate from points of origin to more sensitive sites. Melatonin prevents cataract formation, thereby reducing oxidative damage not only to lipid but also to protein [24].

N-acetylserotonin, an indole precursor for the biosynthesis of melatonin has recently been identified as a potent antioxidant against free radical stress by Siu et al. [25]. They determined the efficacy of N-acetylserotonin against the $Cu^{1+}$ induced lipid peroxidation in bovine retinal homogenates. In previous reports, working with rat retinal homogenates, Siu et al. [26, 27] have compared, in studies in vitro, the efficacy of melatonin, N-acetylserotonin and pinoline with vitamin E. They found that vitamin E was the more effective antioxidant following by N-acetylserotonin, pinoline and melatonin.

Structure-activity studies comparing the free radical scavenging ability of melatonin with several chemically related molecules showed the importance of the methoxy group at position 5 of the indole nucleus and the N-acetyl group on the side chain [28].

Daniels et al. [29] have reported the effect of melatonin and serotonin as free radical scavengers. These authors have postulated that serotonin exerts its free radical scavenging action in the aqueous phase or at the water membrane interphase, while melatonin protects membrane phospholipids against free radical attack by acting within the lipid bilayer of the membranes, and would thus be ineffective as free radical scavenger on the surface of membranes. N-acetylserotonin has the OH-group bond to C5 and is more water-soluble and it would scavenger free radicals within the aqueous environment.

The ability of melatonin to protect against lipid peroxidation is probably not related to scavenging the peroxyl radical ($LOO^{\cdot}$), but rather to its ability to scavenge the initiating agents such as hydroxyl radical ($^{\cdot}OH$). N-acetylserotonin has a phenolic group that would react with peroxyl radicals. Melatonin only possesses indole hydrogens which are not abstracted by peroxyl radicals. N-acetylserotonin also reduces the rate of peroxidation initiated by the Fenton reaction. The rapid metabolic breakdown of melatonin combined with the high capacity for the accumulation of its precursor N-acetylserotonin in the retina support the hypothesis that N-acetylserotonin is an important component of the protective antioxidant system of the retina [30].

We have previously reported that ascorbate-$Fe^{2+}$ induced lipid peroxidation occur in the ROS membranes isolated from bovine retina. This process could be followed by the increase in light emission, i.e. chemiluminescence, and a substantial modification of the fatty acid composition with a considerable decrease of docosahexaenoic acid (22:6 n-3) [31]. In the present study, we examined the effect of indoleamines. $Fe^{2+}$-ascorbate treated ROS membranes were incubated with different concentrations of either melatonin or N-acetylserotonin. In the presence of ascorbic acid, we observed the generation of light emission products in a time-dependent manner. We have found that the chemiluminescence level, an index of lipid peroxidation of ROS, significantly diminished in the presence of both indoleamines.

The present findings suggest that melatonin and N-acetylserotonin provide protection of the photoreceptors against lipid peroxidation. Although we did not attempt to provide mechanistic explanations in this initial work, our results provide strong evidence that indoleamines can act as intracellular free radical scavengers, protecting ROS membranes exposed to lipid peroxidation from deleterious effects and, thus, may play a significant physiological role as antioxidants protecting the retina during oxidative processes.

In conclusion, the present results demonstrate that melatonin and N-acetylserotonin suppresses the ascorbate-$Fe^{2+}$ induced lipid peroxidation processes in ROS membranes and protects the main PUFA from oxidative damage. In this regard, it is important to note that docosahexaenoic acid was protected more efficiently by N-acetylserotonin than by melatonin. Thus, in the present in vitro experimental conditions, N-acetylserotonin shows the highest antioxidant protection. The ability of melatonin and N-acetylserotonin to reduce oxidative damage in vivo may differ from the observations made in the current in vitro studies.

## Acknowledgments

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP 4097, grant to AC and Secretaría de Ciencia y Técnica, Universidad Nacional de La Plata. We thank César Arcemis for the excellent technical assistance. AC is member of Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Tfcnicas de la República Argentina.

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